REMARKS

Applicants have amended claims 1, 3-5, 7, 9-12, 16, 25-27, 31, 35-36, 42, 44, 47, 49 and-50, deleted claims 19 and 24 and added new claims 51-53 in order to more particularly define the invention taking into consideration the outstanding Official Action which at points is confusing. The Official Action appears to confuse cobalamin and transcobalamin and does not appear to appreciate the significance and difficultly of measuring only the holo-TCII of a sample rather than the total TCII content. There is also reference to "TCII bound cobalamin" in the Official Action even though this term was removed from the claims in the last response. If this amendment does not place the application in condition for allowance, the undersigned attorney would like to conduct an interview with the Examiner and perhaps have the inventor present to examine the technology of the invention in an effort to resolve any outstanding issues. Applicants believe that the presently claimed invention is clearly patentable over the references of record and that the claims are in full compliance with 35 USC 112.

The rejection of claims 1, 3-7, 9-12, 16-20, 24-33, 35-36, 42-44 and 47-50 under 35 U.S.C. 112, second paragraph, as being indefinite has been carefully considered but is most respectfully traversed in view of the amendments to the claims and the following comments.

Applicants wish to point out to the Examiner that five key species are at issue in this application; cobalamin, apo-TCII, holo-TCII, apo-HC and holo-HC. Cobalamin (otherwise known as vitamin B_{12}) is a cobalt-containing cofactor, while Transcobalamin II (TCII) and Haptocorrin (HC) are proteins which bind to cobalamin. When the protein is unbound, it is referred to as "apo-" and when it is bound to the cobalt-containing cobalamin, it is referred to as "holo-". "TCII" is used to refer to "apo-TCII" and "holo-TCII" and "HC" is used to refer to "apo-HC" and "holo-HC". Virtually all of the cobalamin in the blood is present as part of a holo-HC or holo-TCII complex. Significantly more holo-HC is present than holo-TCII.

The key aspect of this case, as currently formulated, is the separation and concentration effect provided by the specific binding ligand for TCII or holo-TCII. In

order to reliably measure holo-TCII at the very low levels (~30 x 10^{-12} M and below) present in samples taken from patients with borderline cobalamin deficiency (see passage bridging pages 5 and 6 of Applicants' specification) highly time-consuming methods such as microbiological assays have previously been necessary. Applicants have, however, overcome this problem by using a method including a specific binding step which has two functions; it separates the TCII component from the HC component and it concentrates the TCII component by at least 3 fold. This was illustrated very clearly in Figure 3 submitted with the last response, copy enclosed. The initial sample is shown containing 4 components; apo-TCII, holo-TCII, apo-HC and holo-HC. The immobilised ligand is seen to remove the TCII components from the sample, leaving the HC components in the original tube. The TCII components are then used to create a concentrated sample in a lesser volume of liquid. The total quantity of holo-TCII remains the same but because the volume is less, the concentration is correspondingly higher.

Because the serum level of holo-TCII is so low and the levels of holo-HC and apo-TCII are significantly higher, a successful specific binding and concentration step will require:

1 A ligand with very high affinity to bind as much of the holo-TCII in the sample as possible and avoid losing it from the assay.

2 A very high specificity because if even a small amount of holo-HC in the sample is captured then the assay result will be significantly affected.

3 A very efficient method of release, so that essentially all of the TCII can be released into a small volume for measurement.

A method for reliably assaying holo-TCII employing a combined separation and concentration step using a specific binding ligand with the above key features has never previously been proposed, let alone achieved in accordance with the presently claimed invention. Previous exemplified methods of separation have used non-specific binders such as silica, which absorb only part of the TCII, are not highly specific and so also absorb some of the holo-HC and do not release the bound TCII quantitatively, so some of the holo-TCII is lost. To the extent that specific binders are suggested, no

exemplification is given and no suggestion is made of a combined separation and concentration step or of a ligand with the above properties (1-3).

Additionally, there are three ways in which the holo-TCII content of the concentrated sample can be measured:

i) Measure the holo-TCII complex itself

ii) Measure the cobalamin portion of the holo-TCII.

iii) Measure the TCII protein portion of the holo-TCII.

This is illustrated in Figure 2 submitted with Applicants' last response, in which only the portion of the original sample containing TCII (horizontal shading) and cobalamin (diagonal shading) remains after the various binding and release steps. A further copy of this figure is submitted herewith for the convenience of the Examiner. Clearly, this could be quantified either by measuring it's TCII protein content, or by measuring the cobalamin it contains, or by using a binder specific to the complex as a whole. Assays suitable for measurement of the TCII protein or holo-TCII part of the complex are described on page 12 of the specification.

In an attempt to clarify the invention further, previous claim 1 has been split into two claims (claims 1 and 51), each relating to one of the embodiments previously covered. Claim 1 is directed to specific binding of holo-TCII only, while claim 51 is directed to specific binding of TCII (ie both apo- and holo-TCII). It is important to note that the key binding and separation step is equivalent, irrespective of which binding ligand is used. The claim language has also been clarified and the dependent claims amended in response to the Examiner's comments. Where dependent claims are suitably dependent upon either of claims 1 or 51, I have included the alternative dependence in a single claim.

In view of the above points, Applicants wish make the following observations on the Office Action, by reference to the Examiner's paragraph numbering:

The Examiner objects that various claims, particularly claim 1 are vague and indefinite. Applicants wish to point out that holo-TCII is a complex of one molecule of the TCII protein and one molecule of the cobalt-containing cofactor cobalamin. As a result, holo-TCII may be "determined", as claimed in claim 1 by measurement in any

of the three ways considered above (i-iii). The key aspect of the current invention is that the concentration of holo-TCII in the body sample is directly correlated to a higher concentration in the "cobalamin containing sample". This correlation is brought about by the specific binding of TCII or holo-TCII and release into a suitably reduced volume.

Amended claim 1 as herewith has been reduced to claim only one embodiment of the two previously recited, with the alternative embodiment placed in new independent claim 51. This amendment has been made for clarity but the scope of the two new claims is equivalent to that of previous claim 1.

It is notable that the Official Action refers to the term "transcobalamin II bound cobalamin" which was deleted from claim 1 in our last response.

The Official Action questions the equivalence of holo-TCII to the measurement of cobalamin content. It is clear from the amended claims 1 and 51, and particularly by reference to Figure 3, that the amount of holo-TCII in the "cell free sample" is equivalent to the amount of cobalamin (and equally to the amount of TCII protein) in the "cobalamin containing liquid". This is the case because the holo-TCII is selectively extracted by the steps recited in the claims and released into a known reduced volume. It is this selective extraction and concentration step which most clearly distinguishes claim 1 from the prior art. Amended claim 1 and new claim 51 have been amended to emphasise this distinction between the "determined" value of the holo-TCII in the "body sample", which is the aim of the method, and the "measured" amount of cobalamin or TCII protein in the "cobalamin containing liquid", which is how this value is established.

Because holo-TCII is a 1:1 complex of cobalamin and the TCII protein, the amount (ie number of moles) of holo-TCII in the cell free sample is the same as the amount of cobalamin released from the specific binding ligand. Whether the cobalamin is bound or unbound at the time of measurement does not alter this value.

If the specific binding ligand has specificity for holo-TCII (as in claim 1, rather than TCII as in claim 51) then only holo-TCII will be present in the "cobalamin containing liquid" and thus the amount of cobalamin will be equal to the amount of TCII protein and both will be equal to the amount of holo-TCII in the "body sample". The purification and concentration effects achieved by the intermediate steps makes the

concentration of these components high enough to measure and relates the final measured value directly to the holo-TCII content of the body sample, which was the aim of the assay.

Where the specific binding ligand is for TCII (ie both apo- and holo-, as in claim 51) the cobalamin present in the cobalamin containing liquid arises solely from that present as holo-TCII in the cell free sample. If a pre-separation step comprising the removal of apo-TCII and apo-HC is made (as in dependent claim 16) then the TCII protein content of the "cobalamin containing liquid" arises solely from that present as holo-TCII in the cell free sample.

Thus, in view of the above, all the method steps required to relate the holo-TCII level in the "body sample" to the measured value of the cobalamin or TCII protein arising from holo-TCII in the "cobalamin containing liquid" are positively recited in the claim. A complete method for assaying holo-TCII levels is provided to a worker of ordinary skill by claims 1 or 51. Accordingly, it is most respectfully requested that this rejection be withdrawn.

The rejection of claims 1, 3-7, 9-12, 16-20, 24-33, 35-36, 42-44 and 47-50 under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention has been carefully considered but is most respectfully traversed.

The Examiner objects to the introduction of "a volume of liquid which is at least 3 limes less than the volume of said cell free sample". This term is clearly an inherent part of the original disclosure because in order to create a liquid having al least 3 times the concentration, it is evident to a skilled worker that only a maximum of one third of the volume can be used:

Concentration = Amount (moles) ÷ Volume

Therefore, since the amount (ie number of moles) of cobalamin released from the specific binding ligand can be no greater than the amount of holo-TCII in the cell free sample, if the concentration is to be 3-fold greater, the volume must decrease by

the same ratio as would be appreciated by one of ordinary skill in the art to which the invention pertains.

Nonetheless, in order to avoid any risk of further objection on this point, the amended claims 1 and 51 now recite, "a volume of liquid so reduced in comparison the volume of said cell free sample". This is clearly functionally limited to proving the 3-fold increase in concentration for which there is ample basis in the application. Accordingly, it is most respectfully requested that this rejection be withdrawn in view of the above comments and amendments to the claims.

The rejection of claims 1, 3-7, 9-12, 16-20, 24-33, 35-36, 42-44 and 47-50 under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for specific binding ligands which bind TC II such as an anti-TC II antibody or anti-TC antibody fragment, does not reasonably provide enablement for a specific binding ligand such as polypeptide, oligopeptide, small organic chemical, binders from combinatorial chemistry libraries or phage display library or specifically binding sequences of DNA and RNA has been carefully considered but is most respectfully traversed in view of the following comments.

The Official Action asserts that the specification contains no enabling disclosure for the use of non-antibody specific binding ligands and believes that there is no evidence that such other ligands exist. Applicants wish to point out, however, that the basis of antibody binding is exactly equivalent to the binding of other species such as oligopeptides, antibody fragments, DNA or RNA oligomers or small organic molecules. Ultimately, binding between two species requires complimentary patterns of charge and topology and if a species has a sufficiently well defined spacial charge pattern then it will be simply a matter of routine to find a complimentary binder of the types suggested. Clearly TCII and holo-TCII have the necessary space/charge fingerprint to allow specific binding because antibodies have been identified. It must therefore only be a case of testing a suitable library of potential binders in order to identify one with a complimentary structure. If a library of suitable oligomers can be made and tested, it is inevitable that one will exist having a structure complimentary to TCII.

An article by Kenan et al. is enclosed for the Examiner's attention. This was published 4 years before the current priority and reviews the way in which molecular "shape" can be exploited to find binders of various types. All of the methods considered in Kenan would have been familiar to a skilled worker and the automated screening methods hinted at were significantly further developed by the priority date of the present application.

As the Examiner is no-doubt aware, antibodies and antibody fragments are themselves polypeptides and so no objection can be realistically made with respect to these. With regard to DNA and RNA oligomers and "phage-display" libraries, these are produced and screened using exactly the same laboratory methods as are used for forming and testing recombinant monoclonal antibodies and antibody fragments. As a result, a worker with the normal skill required to form recombinant antibodies and antibody fragment libraries could routinely make and test libraries of other polypeptides, DNA and RNA.

Although the actual synthesis of combinatorial libraries of organic chemicals can be complex and is outside the core skills of a single worker skilled in biochemical assays, methods for generating such libraries would be available within a typical research and development program and the libraries themselves have even been commercially available for some years. The methods necessary for screening an organic chemical library, such as a "one-bead-one-compound" library on a polymeric support, in order to identify a suitable binder are highly similar to the assays used for identifying monoclonal antibodies with specific binding ability. As a result, a skilled reader would require no more disclosure than that provided in order to reproduce the invention over the whole of the claimed scope.

Applicants most respectfully submit that the claims limited to oligomer-type binders (ie antibodies, peptides, DNA & RNA). Preferably, at least antibodies, antibody fragments and DNA/RNA oligomers should be clearly acceptable.

It is important to note that the ligand is limited to a "specific binding ligand for" TCII or holo-TCII. As a result, just as only one monoclonal antibody in millions will show binding affinity for TCII, equally only one DNA oligomer or small organic molecule in a

very large number will have such specificity. Nonetheless, the claims are limited to those which have this specific binding ability, they inevitably exist and a skilled worker could find examples of each type using only familiar, routine methods without undue experimentation. Such methods have existed for at least 10 years and would require no more detailed teaching than is provided in the specification. Accordingly, it is most respectfully requested that this rejection be withdrawn.

The rejection of claims 1, 3-7, 9-12, 16-20, 24-33, 35-36, 42-44 and 47-50 under 35 U.S.C. 112, second paragraph, as being indefinite has been carefully considered but is most respectfully traversed.

As discussed above, holo-TCII is a 1:1 complex consisting of a protein part (the TCII protein) and a cofactor part (cobalamin) in equal amounts (by mole). It is thus clear that the "TCII-protein content" in a sample is the amount of TCII protein present. In order to avoid any possible ambiguity, the wording of claims 1 and 51 has been amended to read "the amount of cobalamin or TCII protein". Accordingly, it is most respectfully requested that this rejection be withdrawn.

The objection to claims 16-20 and 25 as being unclear has been carefully considered and claim 16 has been amended by the introduction of "wherein said preliminary step is carried out prior to contacting said cell free sample with said specific binding ligand". It is believed that this addresses the Examiner's objection. A similar change has been made to claim 26, since the Examiner's comment presumably applies equally to this claim.

The objection to claims 16 and 19 wherein the Examiner states it is unclear whether the apo forms are comprised with the same or added to the sample has been carefully considered. Claim 16 now makes it clear that the apo-HC and apo-TCII referred to are those present in the cell free sample. Claims 19 and 24 have been deleted.

The objection to claim 19 as being unclear has been obviated by the cancellation of claim 19.

The objection to claim 24 as being unclear has been obviated by the cancellation of claim 24.

The objection to claim 25 as being inconsistent has been obviated by the amendment to the claim and in view of the following comments. Claim 25 has been clarified by indicating that at least 80% of the holo-TCII from the cell free sample is bound to the specific binding ligand and therefore is present in the ligand bound fraction.

The indication that claims 35 and 36 are unclear has been obviated by the amendment to the claims. Further, as discussed above, the cobalamin concentration is determined by the amount of holo-TCII present in the sample and the relative volumes of the cell free sample and the cobalamin containing liquid. It is believed that the changes made to claims 1 and 51 render claims 35 and 36 entirely clear in scope. Reference is again made to Figure 3 submitted with the last response. It is most respectfully requested that these objections to the claims be withdrawn in view of the foregoing comments and amendments to the claims.

The rejection of claims 1, 5, 7, 9, 11, 12, 42-44, 47, 48 and 50 under 35 U.S.C. 102(b) as being anticipated by Herbert et al. has been carefully considered but is most respectfully traversed in view of the amendments to the claims and the following comments.

This section of the Office Action is based (nearly word for word) on section 14 of the 13 April 1999 Office Action and section 6 of the 10 October 2000 Office Action. On the previous occasions, the rejection was on the grounds of obviousness with regard to various claims from claim 8 onward, when combined with two other citations. Herbert was not raised as an issue against the earlier claims. Claims 1, 5 and 7 are now also rejected over this citation (this time alone) on the grounds of anticipation. Applicants' amendments to claim 1 have only made this more specific and thus it is very difficult to address the rejection when it is so inconsistent in nature.

Applicants wish to direct the Examiner's attention to MPEP § 2131 which states that to anticipate a claim, the reference must teach every element of the claim.

> "A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). "The identical

> invention must be shown in as complete detail as is contained in the ... claim." *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed Cir. 1989). The elements must be arranged as required by the claim, but this is not an *ipsissimis verbis* test, i.e., identity of terminology is not required. *In re Bond*, 910 F.2d 831, 15 USPQ2d 1566 (Fed.Cir. 1990).
>
> *Akzo N.V. v. International Trade Comm'n*, 808 F.2d 1471, 1 USPQ2d 1241 (Fed. Cir. 1986) (Claims to a process for making aramid fibers using a 98% solution of sulfuric acid were not anticipated by a reference which disclosed using sulfuric acid solution but which did not disclose using a 98% concentrated sulfuric acid solution.).

Applicants wish to respond to the Official Action by pointing out the key features of claims 1 and 51 which are neither disclosed nor taught by Herbert. In particular, the claim requires:

i) The **specific** binding of the TCII (claim 51) or holo-TCII (claim 1) component

ii) The **concentration** of this component into a smaller volume for analysis.

The key separation and concentration effect can only be achieved by having a ligand which binds with very high affinity and is very selective for TCII over HC. Herbert completely fails to appreciate this point. The required concentration effect will be much more difficult to achieve if the ligand fails to bind virtually all of the holo-TCII from the sample because the original amount is very small and the remainder will be wasted. The assay will also be meaningless if even a few percent of the holo-HC is bound because holo-HC is present in the original sample in a much higher concentration and could swamp the assay. Because only 25% or less of the cobalamin exists as holo-TCII and the serum concentration is at or below the limit of cobalamin detection, exceptional demands are made upon a specific binding ligand and the non-specific ligands recommended by Herbert are simply not good enough.

The physico-chemical absorption carried out by Herbert is known not to bind all of the TCII in a sample and to take up some of the HC. If Herbert had intended a concentration step it would have been essential to address this issue and he does not.

The only context in which antibodies are indicated is as a substitute for silica and they are not preferred in Herbert's method because silica is readily available. There is no motivation to use antibodies in Herbert's method because they are more difficult and expensive to obtain and Herbert indicates no advantage that they might offer and there is clearly no anticipation. It is only by appreciating the value of a concentration step that the need for specific binding ligands is made clear and there is absolutely no teaching towards this in any of the prior art.

As indicated in the specification (pages 5-6) and supported in the accompanying articles by Wickramasinghe (J. Clin. Pathol. **49** (1996), 755) and by Carmel (Clin. Chem. **48** (2002), 407), the assay method used by Herbert to detect the extracted cobalamin is effective only at concentrations down to 40 pM (around 30 pg/ml). Since Herbert dilutes the sample 5-fold in the Example, the assay is only effective down to a serum concentration of 200 pM. This may partially explain why the values for healthy individual #4 fail to tally by around 30 pg/ml.

It is evident that Herbert was unaware that his method could not reliably detect cobalamin at the level required and did not appreciate that the physico-chemical binders recommended in his method were not highly efficient or specific. It is only in light of the current specification that any method capable of such analysis in a routine and straightforward way has been revealed. This is in spite of more than a decade of attempts between Herbert and the current application. It is only in hindsight, with knowledge of the current application that such a method is available and it is clearly not inherent in the Herbert reference.

Herbert teaches the equivalence of antibodies and non-specific methods such as silica absorption. This clearly teaches away from a method including specific binding and concentration because silica is completely unsuitable for this. The extraction of holo-TCII with a specific binding ligand and the concentration of this extract into a lower (known) volume are both requirements of claims 1 and 51 and are neither disclosed nor taught in Herbert without prior knowledge of the current application.

Claim 1 as herewith amended is even further removed from any teaching of Herbert. This claim requires the use of specific binding ligands that not only have very

high affinity and a very high level of specificity, but can also distinguish between holo-TCII and apo-TCII. There is nothing in Herbert to indicate that such binders were envisaged and indeed they would offer no advantage in Herbert's method. A skilled worker would therefore have no motivation to use holo-TCII specific binders in the method of Herbert. Accordingly, it is most respectfully requested that this rejection be withdrawn.

The rejection of claims 3, 16, 17, 24-26 and 35-36 under 35 U.S.C. 103(a) as being unpatentable over Herbert et al., in view of Houts et al. has been carefully considered but is most respectfully traversed.

The Examiner repeats the section bridging pages 8 and 9 of the 13 April 1999 Office Action, to which responses were filed in July 2000 and December 2001. There is no indication that these detailed responses have been considered and are herein incorporated by reference.

The first three lines of the discussion of Houts is largely accurate but is not relevant to current claim 1. The method described is a cobalamin detection method, which might, for example, be employed to measure the cobalamin content of the "cobalamin containing liquid" as required in claim 1. It is the sample preparation steps of specific binding and concentration which are key to the current invention and Houts makes no disclosure which could be considered relevant to these. This also applies to the remainder of section 15, except where the Examiner has misinterpreted Houts, as described below.

The statement "Also, competitive binding assays use proteins which not only bind B_{12} but also cobalamin analogues including transcobalamin II and, R-proteins and intrinsic factor" is clearly a misunderstanding and is specifically traversed. As previously discussed, cobalamin is a small, cobalt-containing cofactor (molecular weight ~1355), which enables the functioning of certain enzymes such as methyonine synthase. In contrast, TCII, R-proteins and intrinsic factor are all proteins which bind to cobalamin at various stages of cobalamin absorption and distribution in the body. These proteins cannot be cobalamin analogues. The analogues to which Houts refers are so-called "cobalamin metabolites" which exist naturally in the blood but are non-functional as

enzyme cofactors. These non-functional analogues are bound by some cobalamin proteins such as R-binders but not by intrinsic factor or by TCII. Houts refers to the binding of analogues as a disadvantage of proteins such as R-binder in comparison with intrinsic factor, which is specific for active B_{12}.

The statement that "The cited references teach a separation step and steps that release...previously bound cobalamin into a concentrated environment" is simply inaccurate and respectfully traversed. The only reference to volumes in Houts (column 5 lines 7-31) discloses the dilution of the original sample 6.5-fold. The centrifugation step described in Houts removes excess label from the solution, before the liquid phase is removed for analysis. The albumin-coated charcoal which is removed by the centrifugation has no relevance to the assay method except as a way of removing excess radiolabel. This step achieves no concentration of the analyte and no concentrated sample is formed.

The Examiner refers to a "competitive sandwich ELISA assay on solid support" but no discussion of ELISA methods is present in Houts. Accordingly, it is most respectfully requested that this rejection be withdrawn.

The rejection of claims 6-7, 12 and 8-20 under 35 U.S.C. 103(a) as being unpatentable over Herbert et al. and Houts as applied to claims 1, 5 and 16 above further in view of McLean et al. has been carefully considered but is most respectfully traversed.

This section is largely based on section 13 of the 13 April 1999 Office Action and detailed explanations and arguments were presented in the several previous responses and are herein incorporated by reference.

In summary, McLean uses antibodies against TCII to block the uptake of B_{12} into cells. The effects of these antibodies in starving cells of cobalamin are shown to be reduced by applying very high concentrations of free cobalamin, which is absorbed into the cells by non-specific uptake. This document has no relevance to an assay method for holo-TCII, except in that it demonstrates the existence of specific binding ligands.

There is nothing in either Herbert of McLean that would lead the two to be combined because McLean is investigating a completely different subject and Herbert

indicates no benefit in the use of antibodies rather than silica. Neither document makes any suggestion that a specific binding ligand could be used to concentrate the holo-TCII in a body sample. The combination of either or both of these with Houts adds no teaching towards the invention because Houts relates to a cobalamin detection step which is applicable only after the key specific binding and concentration steps of the invention. Furthermore, the assay for antibody "panning" described by McLean detects entirely the protein component of TCII and Herbert assays only the cobalamin content, the two cannot be combined to give a holo-TCII assay.

The Examiner does not address the issue of the appreciating that highly specific binders can be used to provide a simultaneous separation and concentration step which allows the level of holo-TCII to be measured. The method of Houts could not be used to measure the cobalamin content of separated holo-TCII in the absence of a concentration step and no such step is indicated in Herbert or McLean. Therefore, even in combination, these three publications could not be read to provide the assay of the present invention. It is therefore most respectfully requested that this rejection be withdrawn.

The rejection of claims 4 and 49 under 35 U.S.C. 103(a) as being unpatentable over Herbert et al. in view of Houts and further in view of Allen et al. has been carefully considered but is most respectfully traversed.

This section is taken from section 11 of the 13 April 1999 Office Action and was responded to fully at that time and that response is herein incorporated by reference.

In summary, the method of Allen detects total cobalamin and not holo-TCII. The method is not sufficiently sensitive to detect holo-TCII at levels approaching those present in patients with borderline cobalamin deficiency and therefore cannot provide an automated assay for holo-TCII. The methods of the other citations can only be combined with Allen to give an automated holo-TCII assay if they provide a method for assaying holo-TCII at biologically significant levels and they do not. It may be obvious to provide an automated assay for cobalamin based on Herbert, McLean and Allen, but that is not what is claimed in claims 1 and 51. The present claims relate to a method for assaying holo-TCII and even in combination, the cited documents are incapable of

providing this. Accordingly, it is most respectfully requested that this rejection be withdrawn.

The rejection of claims 27-33 under 35 U.S.C. 103(a) as being unpatentable over Herbert et al. and McLean et al. as applied to claims 1 above further in view of Hoyle et al. has been carefully considered but is most respectfully traversed.

The Examiner cites Hoyle and states that claims 27-33 are obvious in light of this, in combination with Herbert and McLean.

The disclosure in Hoyle relates to antibodies raised to bind cobalamin (not antibodies against TCII). These are used as a substitute for intrinsic factor, which was not available in pure form at the time Hoyle was published (column 1 line 63 to column 2 line 9). Such antibodies would be useful only in the final step of the present invention, if the cobalamin content of the "cobalain containing liquid" was to be measured. This occurs after the key concentration and separation step.

The Examiner believes that the affinities claimed are "conventional" for monoclonal antibodies, but in fact, Hoyle describes antibodies having binding affinities of 5×10^9 l/mol or greater as exceptional (column 6 lines 9 to 14). This is in line with the common knowledge in the art that typical "conventional" affinity constants range from 10^7 to 10^9 l/mol.

The teaching of Hoyle therefore adds nothing relevant to the creation of antibodies of high affinity against TCII or holo-TCII. Hoyle demonstrates that cobalamin itself has suitable surface charge and topology to allow exceptionally strong antibody binding but when cobalamin is bound to TCII, this surface will be unavailable. It is the surprising discovery of the inventors that TCII has surface properties allowing exceptionally strong antibody binding and this could not be inferred from Hoyle. It is therefore most respectfully requested that this rejection be withdrawn.

As indicated in the Official Filing receipt and reflected in the USPTO date base, this application is a continuation of parent application serial number 09/271,764. This cross reference to the parent application in the continuation transmittal papers has been updated to reflect the status of the parent application as abandoned. Confirmation of the claim for domestic priority , box 15 on the Summary sheet, in the next Official Action

is most respectfully requested. Similarly, an acknowledgment of the claim for priority under 35 USC 119 and receipt of the priority document filed on January 29, 2001, is most respectfully requested.

In view of the above comments and further amendments to the claims, favorable reconsideration and allowance of all of the claims now present in the application are most respectfully requested.

Respectfully submitted,

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MARKED-UP VERSION SHOWING CHANGES MADE

IN THE SPECIFICATION:

Please replace the first sentence of the specification with the following amended first sentence.

This application is a continuation of nonprovisional application serial number 09/201,764 filed December 1, 1998, now abandoned.

IN THE CLAIMS:

Please replace claims 1, 3-5, 7, 9-12, 16, 25-27, 31, 35-36, 42, 44, 47, 49 and-50 with the following amended claims.

1(Fourth amended). An assay method for the determination of holo-transcobalamin II (holo-TC II)in a body sample, comprising contacting a cell free sample of a body fluid with an immobilised or immobilizable specific binding ligand for [transcobalamin II (TC II) or] holo-transcobalamin II (holo-TC II), whereby to form bound holo-TCII, separating a ligand bound fraction from a non-ligand bound fraction, releasing said bound holo-TCII from the ligand bound fraction into a volume of liquid so reduced in comparison with the volume of said cell free sample, to provide a cobalamin containing liquid having a cobalamin concentration at least 3 times the holo-TCII concentration in said cell free sample, and determining the holo-TCII content in said body sample by measuring the amount of cobalamin or TCII-protein in said cobalamin containing liquid arising from the bound [cobalamin in the ligand bound fraction into a volume of liquid which is at least 3 times less than the volume of said cell free sample, to provide a cobalamin containing liquid wherein the cobalamin concentration is at least 3 times the holo-TC II concentration in said cell free sample of body fluid and determining the cobalamin content in said cobalamin containing liquid by measuring the cobalamin or TCII-protein content arising from the] holo-TCII released from the specific

binding ligand and relating this to the volumes of said cobalamin containing liquid and said cell free sample.

3(Amended). An assay method as claimed in claim 1 or claim 51 wherein said assay is capable of detecting holo-TC II in said body sample at a concentration as low as 9 pM.

4(Thrice Amended). An assay method as claimed in claim 1 or claim 51 wherein said assay is [performed] effected to analysis by an automated process.

5(Twice Amended). An assay method as claimed in claim 1 or claim 51 wherein said specific binding ligand is selected from the group consisting of polyclonal and monoclonal antibodies, antibody fragments, polypeptides, oligonucleotides, small organic chemicals, specific binders selected from combinatorial chemistry libraries, specific binders selected from phage display libraries, specifically binding sequences of DNA, and specifically binding sequences of RNA.

7(Amended). An assay method as claimed in claim 1 wherein said specific binding ligand exhibits a high degree of selectivity [and specificity] towards [TC II] holo-TCII and exhibits low affinity towards other [TC proteins, in either apo or halo form, or any other] cobalamin binding proteins, in either apo or holo form.

9(Twice Amended). An assay method as claimed in claim 1 or claim 51 wherein said [cobalamin] bound holo-TCII is released by changing the temperature or the pH of the surrounding medium.

10(Amended). An assay method as claimed in claim 1 or claim 51 wherein the different cobalamin forms are converted to the less light sensitive cyanocobalamin by treatment with KCN prior to contacting said sample with a specific binding ligand.

11(Amended). An assay method as claimed in claim 1 or claim 51 wherein [said free cobalamin is determined] the cobalamin content of said cobalamin containing liquid is measured by a competition assay performed by contacting an immobilised binding partner for cobalamin with the dissociated cobalamin of the sample in the presence of labelled ligand which competes with the isolated cobalamin for binding to the immobilised binding partners.

12(Amended). An assay method as claimed in claim [1] 51 wherein the binding ligands for TC II are immobilised and bind to both holo- and apo-TC II.

16(Amended). An assay method as claimed in claim [1] 51 wherein a preliminary separation step is carried out using an immobilised or immobilisable cobalamin or an analogue or fragment thereof which selectively binds the apo-forms of both TC II and haptocorrin (HC) contained within said cell free sample, such that the apo forms of the TC II and HC proteins are bound by the cobalamin, analogue or fragment thereof and separated from the holo-TC II and holo-HC complexes, wherein said preliminary step is carried out prior to contacting said cell free sample with said specific binding ligand.

25(Twice Amended). An assay method as claimed in claim 1 wherein at least 80% of holo-TC II present within said cell free sample is contained within said ligand bound fraction.

26(Twice Amended). An assay method as claimed in claim 1 or claim 51 further comprising a preliminary separation step in which the cell free sample is contacted with an immobilized or immobilizable specific binding ligand for haptocorrin wherein said preliminary step is carried out prior to contacting said cell free sample with said specific binding ligand.

27(Amended). An assay method as claimed in claim [1] 51 wherein said TC II binding ligand possesses an affinity constant of at least $10^9 M^{-1}$.

31(Amended). An assay method as claimed in claim 1 or claim 51 wherein the degree of cross reactivity of said specific binding ligand with HC is less than 1%.

35(Twice Amended). An assay method as claimed in claim 1 or claim 51 wherein the [binding ligands for TC II or holo-TC II concentrate the ligand by at least 5-fold] concentration of cobalamin in said cobalamin containing liquid is at least 5-fold greater than the concentration of cobalamin in said sample.

36(Amended). An assay method as claimed in claim 1 or claim 51 wherein the [binding ligands for TC II or holo-TC II concentrate the ligand by at least 10-fold] concentration of cobalamin in said cobalamin containing liquid is at least 10-fold greater than the concentration of cobalamin in said sample.

42(Twice Amended). An assay method as claimed in claim 1 or claim 51 wherein said body sample is selected from the group consisting of seminal fluid, cerebro-spinal fluid, amniotic fluid and blood derived samples.

44(Amended). An assay method as claimed in claim 1 or claim 51 wherein said bound fraction is separated from said unbound fraction by precipitation, centrifugation, filtration or chromatographic methods.

47(Amended). An assay method as claimed in claim 1 or claim 51 wherein said [the binding ligands used to separate a fraction of a sample are immobilised on a particulate solid phase support] specific binding ligands are immobilised on a particulate solid phase support.

49(Twice Amended). A kit for use in a diagnostic assay according to claim 1 or claim 51, comprising:

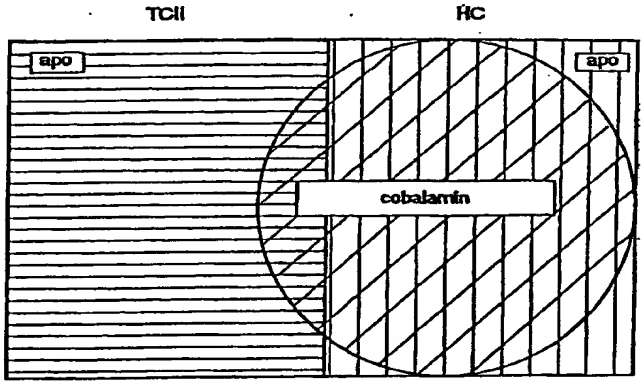
an immobilized or immobilizable specific binding ligand for TC II or holo-TC II;

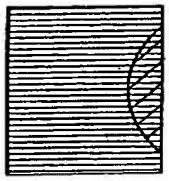
a plurality of holo-TC II solutions of known concentration;

a release agent to release cobalamin from holo-TC; and

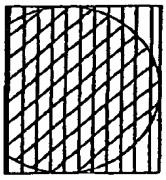
optionally a labelled ligand

50(Amended). An assay method as claimed in claim 1 or claim 51 wherein the cobalamin content in said cobalamin containing liquid is determined in a competitive binding assay.

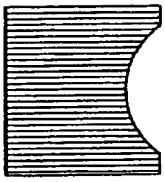




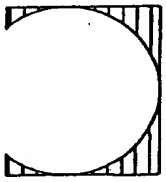
TCII (Transcobalamin II)



HC (Haptocorrin)

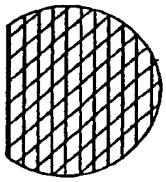


apo-TCII (TCII with no cobalamin)

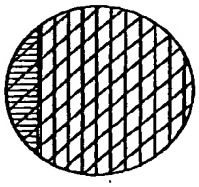


apo-HC (HC with no cobalamin)

holo-TCII (TCII with cobalamin bound)
(Biologically functional cobalamin)



holo-HC (HC with cobalamin bound)



Total cobalamin (= holo-TCII +holo-HC)

Figure 1

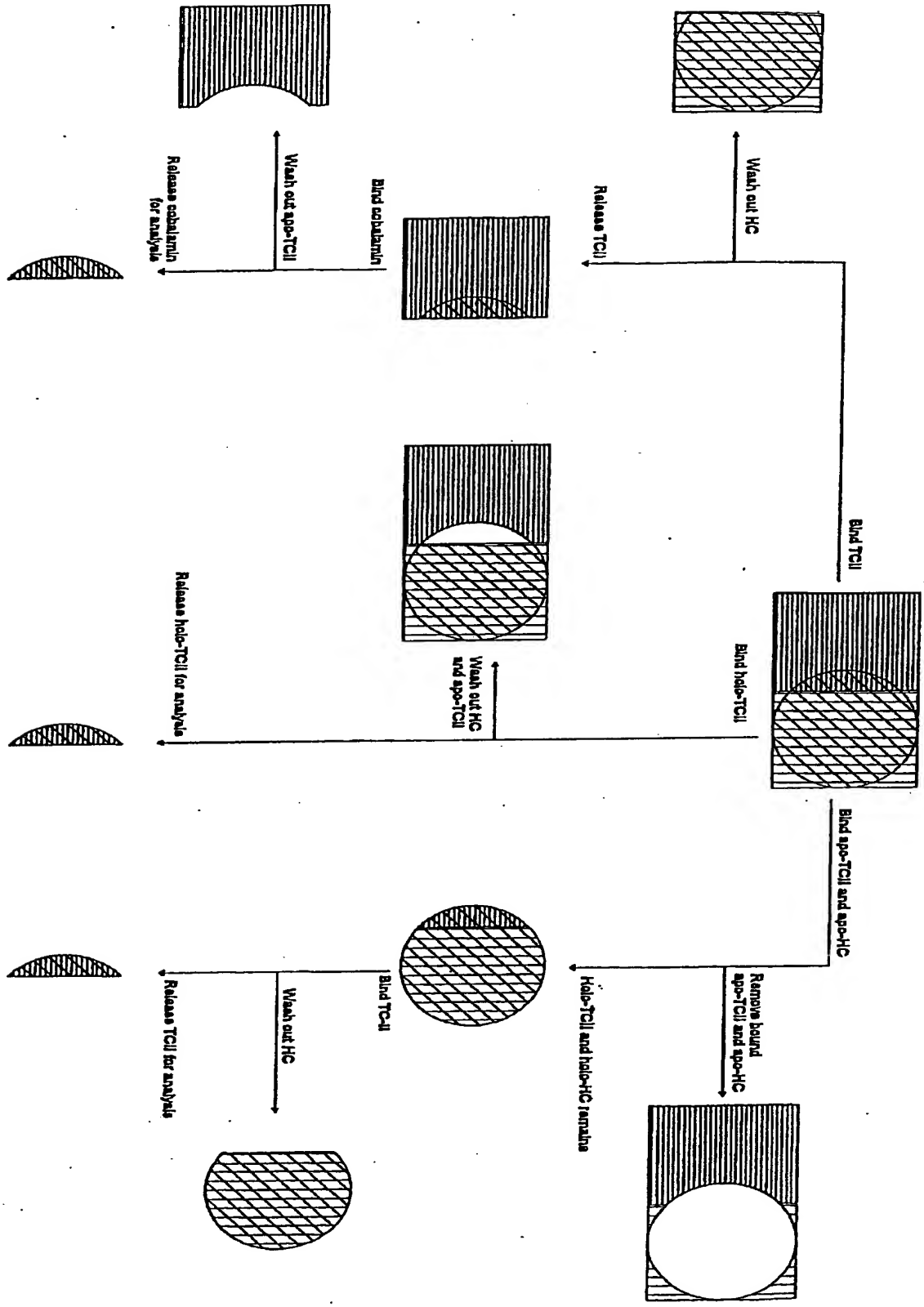

Wash out HC
Release TCII
Bind TCII
Bind holo-TCII
Wash out HC and apo-TCII
Release holo-TCII for analysis
Bind apo-TCII and apo-HC
Remove bound apo-TCII and apo-HC
Holo-TCII and holo-HC remains
Bind TC-II
Wash out HC
Release TCII for analysis
Bind cobalamin
Wash out apo-TCII
Release cobalamin for analysis

Figure 2

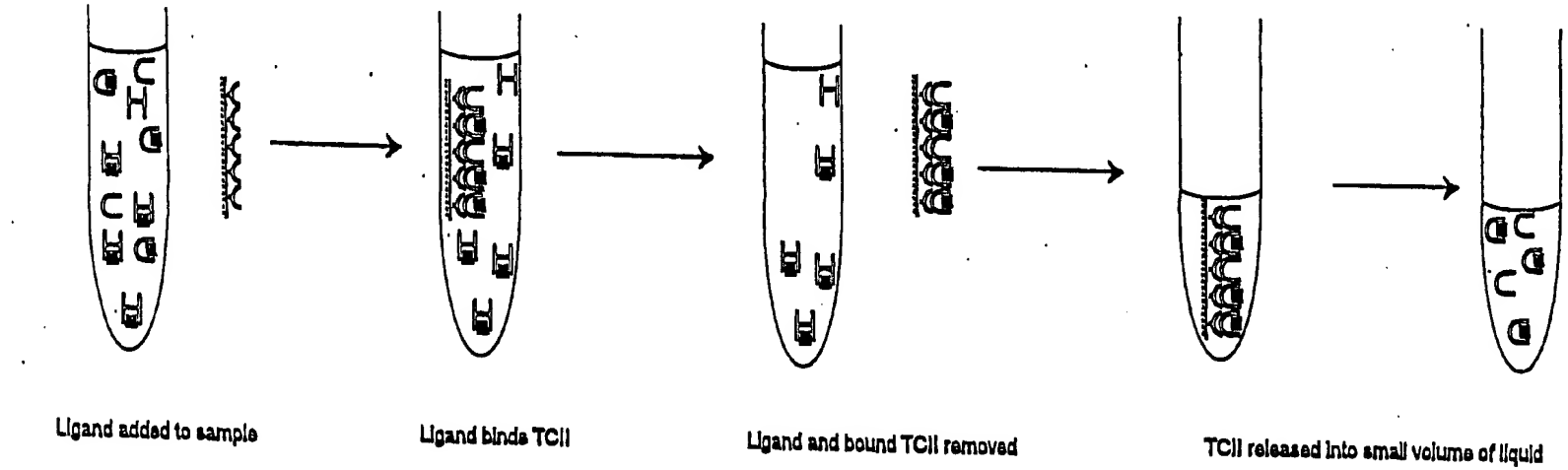


Figure 3

Measuring and Interpreting Holo-Transcobalamin (Holo-Transcobalamin II)

At one time, diagnosing cobalamin deficiency was fairly simple, usually involving a patient with clinical problems, and was usually settled by determining whether the serum cobalamin concentration was low or not. As reviewed elsewhere *(1)*, things began to change after sensitive metabolic tests were introduced and as attention extended to the cobalamin status of asymptomatic persons. Metabolic studies confirmed that most low cobalamin concentrations in asymptomatic patients and seemingly healthy persons represented subclinical cobalamin insufficiency, but ~30–40% of these cobalamin concentrations did not represent insufficiency and thus could be considered "falsely low". The diagnostic reliability of serum cobalamin was further challenged by metabolic demonstrations of "falsely normal" cobalamin concentrations; these too occurred most often, but not exclusively, in asymptomatic persons.

The search continues for the optimal test to diagnose deficiency because the metabolic tests also have disadvantages. Increased plasma total homocysteine is too nonspecific; methylmalonic acid determination, although posing fewer problems of specificity, is complex and expensive; and the deoxyuridine suppression test is too unwieldy for practical use.

Interest has been drawn to the possible benefit of measuring only the cobalamin attached to transcobalamin (TC; also called TC II; the TC-cobalamin complex is called holo-TC or holo-TC II) rather than the total cobalamin content of plasma. The concept, suggested by Lindemans et al. in 1983 *(2)*, is simple and attractive: holo-TC contains the biologically available cobalamin because only TC promotes specific uptake of its cobalamin by all cells. The much larger fraction of serum cobalamin carried by haptocorrin (HC; also called TC I, R binder, or sometimes cobalophilin) is considered metabolically inert because no cellular receptors exist for holo-HC (also called holo-TC I).

However, the physiologic cycle of holo-TC is quite complex: (*a*) ileal holo-TC enters the portal circulation, carrying cobalamin absorbed from the gut *(3)*; (*b*) some of the portal holo-TC is cleared by hepatocytes, with holo-TC concentrations decreasing by 26–72% in the hepatic vein *(4)*; (*c*) the uncleared holo-TC then circulates systemically, where an unknown proportion is delivered to other cells; (*d*) most of the peripheral holo-TC clearance occurs via glomerular filtration followed by tubular uptake in the kidney, which is very rich in cobalamin and whose tubular epithelium is rich in TC receptors *(5)*; (*e*) animal data suggest that renal tubules also synthesize TC, which emerges in the blood stream as new holo-TC carrying the reabsorbed cobalamin *(6)*. The pathologic influences on most of these individual phases in the cycle are still unknown.

Many things affect the holo-TC concentration found in antecubital vein blood. These include not only the amount of absorbed cobalamin but also the rates of hepatic and renal uptakes of holo-TC, the production and release of ileal and possibly renal holo-TC, tissue requirements for cobalamin, and perhaps other unknown factors such as qualitative and quantitative variations in TC. It also seems worth pondering why 5′-deoxyadenosylcobalamin makes up more of the cobalamin in holo-TC than in holo-HC *(7)*.

It is not surprising, therefore, that what low holo-TC concentrations really tell us remains elusive. The favored hypotheses have been that low holo-TC is either an early sign of general cobalamin insufficiency or specific evidence of decreased absorption of cobalamin. The distinction between these two very separate explanations has blurred, particularly as advocacy became more enthusiastic, but it is not an idle distinction. This central issue and many other questions need resolution.

Malabsorption and deficiency are not identical, nor are their clinical implications. Patients can have cobalamin deficiency without having malabsorption and can have malabsorption without deficiency. If holo-TC concentrations reflect absorption alone, then even the transient malabsorption caused, for example, by temporary exposure to drugs, such as colchicine or omeprazole, or by alcohol abuse should depress holo-TC. Moreover, if holo-TC concentrations depend primarily on the influx of absorbed cobalamin, temporary dietary restriction might also depress holo-TC, although indirect evidence from two old studies suggests otherwise *(2, 8)*. Either of these types of temporary influences could limit the clinical and metabolic value of the test because it takes years to deplete cobalamin stores.

Does the evidence favor cobalamin deficiency or impaired absorption as the determinant of low holo-TC? Convincing evidence for either explanation does not exist today. Support for low holo-TC as a marker of cobalamin malabsorption has rested on data from only four patients with AIDS *(9)* and a troubling study in which gastric and, even more speculatively, duodenal histology was used instead of, and sometimes in conflict with, direct absorption testing to define a state "compatible with malabsorption" *(10)*. Moreover, 20 of the 53 low holo-TC concentrations (38%) occurred in patients who were not in such a state *(10)*. New evidence of low holo-TC concentrations in Indian vegetarians *(11)* suggests that a popular characterization of holo-TC assay as a surrogate Schilling test is incorrect. However, studies equating low holo-TC with

cobalamin insufficiency are also open to question. Future study models must be selected carefully to allow the influences of deficiency and malabsorption to be distinguished clearly. Both malabsorption and metabolic status must be defined rigorously and demonstrated directly rather than by proxy measures.

Specificity of the low holo-TC concentration. Wickramasinghe and Ratnayaka *(12)* concluded that low holo-TC was not specific for cobalamin abnormality. Nine of their 24 patients with low holo-TC (38%) had normal cobalamin status, as determined with the sensitive deoxyuridine suppression test; the diagnoses in the 9 included myelodysplasia, congenital dyserythropoietic anemia, and alcohol abuse. Another study mentioned low holo-TC concentrations in patients with folate deficiency *(10)*.

Clinical utility of holo-TC measurement. Utility will depend ultimately not just on the diagnostic meaning and technical performance characteristics of holo-TC measurement but also on how often and when holo-TC determination clinically outperforms the simple and inexpensive measurement of total cobalamin in the blood. Demonstrations of significant correlations between holo-TC and cobalamin status or absorption will not suffice without also comparing them directly with total cobalamin concentrations. Furthermore, if the preponderant diagnostic advantage of holo-TC testing turns out to be in persons with subclinical cobalamin deficiency, that too must inform judgment about the assay's utility.

Methodologic issues. Technical problems have been major impediments to the resolution of the previously mentioned issues. Plasma contains ~1.5 times as much TC as HC, but only a small fraction of the TC carries cobalamin. Holo-TC was 20–220 pmol/L in a study using immunoadsorption techniques, whereas total TC (apo and holo forms combined) was 422–1086 pmol/L *(2)*. In comparison, holo-HC was 87–491 pmol/L in a total HC of 154–750 pmol/L. Widely quoted estimates are that 6–20% of cobalamin is carried in plasma as holo-TC, although reported values have varied between 0% and >50%.

Holo-TC measurement involves two sequential steps: separation of total TC from total HC [and other minor cobalamin-carrying proteins *(4)*], and determination of the cobalamin content in the TC fraction. Each step has been dogged by technical problems. Separation of TC from HC has relied most often on physical adsorption differences between the two proteins, using adsorbents such as microfine silica *(9, 10, 12)*, which however adsorbs only 85–90% of the TC and adsorbs some HC *(12)*. Other approaches have used immunoadsorption *(2, 8)* or liquid chromatography *(4)*, which is unwieldy. The difficulty of the second step, measuring cobalamin in holo-TC, has been the need for assays to distinguish minute concentrations of cobalamin in holo-TC that are often beyond their precision; a difference between 20 and 25 pmol/L may be diagnostically crucial. That laboratories and assay methods often cannot reliably quantify serum total cobalamin concentrations <50 ng/L (37 pmol/L), instead reporting them all as <50 ng/L, illustrates the difficulty of applying such assays to holo-TC measurement. The usual indirect resort by holo-TC methods to quantifying the larger holo-HC fraction instead and subtracting it from the separately measured total cobalamin value is just as problematic because the CV of the cobalamin assay often exceeds the small difference between the two values.

The two new holo-TC assay methods reported in this issue of *Clinical Chemistry* have addressed many of the technical problems. Both assays use specific anti-TC antibody rather than imprecise physicochemical methods to separate TC from HC and other holoproteins. Ulleland et al. *(13)* also reduced the cobalamin measurement imprecision by concentrating the final sample eightfold, so that the amount of holo-TC cobalamin presented for assay is greater. Nexo et al. *(14)* have used the ingenious approach of reversing the order of manipulations and avoiding the cobalamin assay entirely. They first separated holoproteins from apoproteins by use of cobalamin-coated magnetic beads and then directly assayed the TC fraction of the holoproteins by ELISA. It may be that they thus also avoided the interference by very high apo-TC concentrations in quantifying holo-TC that Ulleland et al. *(13)* found. It is not clear why the assay of Nexo et al. *(14)* measured higher holo-TC concentrations than that of Ulleland et al. *(13)*. Future studies should clarify the performance characteristics of these two methods at the critical juncture between low and normal holo-TC values. It also appears that separate reference intervals will be needed for serum and plasma.

Armed with these new methods, investigators can now address the many questions surrounding holo-TC and what it means, and carefully designed clinical surveys can be undertaken. Until the answers emerge, we must all take very seriously the American commercial distributor's warning that the newly released reagent set, described here by Ulleland et al. *(13)*, is for research purposes only.

To paraphrase the psychiatrist's invitation in the final line of Philip Roth's *Portnoy's Complaint*: And now, perhaps, we may begin?

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Limited value of serum holo-transcobalamin II measurements in the differential diagnosis of macrocytosis

S N Wickramasinghe, I D Ratnayaka

Abstract

***Aim*—To study the value of serum holo-transcobalamin II (holo-TCII) measurements in the differential diagnosis of macrocytosis.**

***Methods*—Holo-TCII concentrations were measured in serum samples from 50 healthy non-vegetarian subjects and 30 patients with macrocytosis, using a technique based on the adsorption of holo-TCII with amorphous, precipitated silica. Deoxyuridine (dU) suppression tests were performed on the bone marrow cells of all the patients. Haematological diagnoses were made using standard criteria.**

***Results*—The causes of macrocytosis were cobalamin (Cbl) deficiency due to pernicious anaemia or following partial gastrectomy (10 patients), dietary folate deficiency with/without Cbl deficiency (four patients), chronic alcoholism (four patients), myelodysplastic syndrome (five patients), treatment with methotrexate or azathioprine (three patients), and congenital dyserythropoietic anaemia (CDA) (four patients). Undetectable or low holo-TCII concentrations were found in all patients with Cbl deficiency and in some or all patients from each of the other diagnostic categories. There was also no correlation between the dU suppressed value and the holo-TCII concentration: all 15 patients with high dU suppressed values and nine of 15 with normal dU suppressed values, including four patients with CDA, had low holo-TCII concentrations.**

***Conclusions*—Measurements of serum holo-TCII concentrations by the silica adsorption method are not of value in the differential diagnosis of macrocytosis. The finding of low serum holo-TCII concentrations in patients with macrocytosis due to causes other than Cbl deficiency may result not only from a state of negative Cbl balance but also from other factors, such as increased utilisation of holo-TCII as a consequence of erythroid hyperplasia.**

(*J Clin Pathol* 1996;49:755–758)

Keywords: holo-transcobalamin II, macrocytosis, deoxyuridine suppressed value.

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About 6–20% of the cobalamin (Cbl, vitamin B_{12}) in serum is bound to transcobalamin II, the Cbl binding serum protein involved in delivering Cbl to cells.[1–3] Normally, only some of the TCII molecules contain bound Cbl and such molecules are termed holo-TCII. It has been proposed that a reduction in the serum holo-TCII concentration may occur as an early sign of negative Cbl balance in the absence of other biochemical, haematological or neurological manifestations of Cbl deficiency.[4 5] In a recent study none of 13 patients with normal total serum Cbl and low holo-TCII concentrations and only nine of 30 patients with a low total serum Cbl and a low holo-TCII concentration were considered to show the effects of tissue Cbl deficiency.[6] The present study was undertaken to determine the value of holo-TCII measurements in the differential diagnosis of macrocytosis.

Methods

Thirty patients with macrocytosis who underwent bone marrow aspiration during the course of their investigation were studied. Macrocytosis was diagnosed on the basis of mean corpuscular volume values determined using a Technicon H2 analyser (Bayer Diagnostics, Basingstoke, UK). Red cell folate concentrations were determined using the Becton Dickinson folate (^{125}I) radioassay kit. In addition to other standard laboratory investigations the following special investigations were carried out.

DEOXYURIDINE SUPPRESSION TEST

An aliquot of freshly aspirated bone marrow was mixed with heparinised Hanks' solution and used to determine the deoxyuridine suppressed value using the method described by Matthews and Wickramasinghe.[7]

HOLO-TCII MEASUREMENTS

Serum holo-TCII concentrations were measured in 50 healthy, non-vegetarian adults and in the 30 patients. A modification of the method of Das *et al*[8] based on the adsorption of holo-TCII by silica was used, except that total and holo-TCII depleted serum Cbl concentrations were determined by the IM_X Cbl assay using the IM_X system (Abbot Diagnostics Division, Maidenhead, Berks, UK). The IM_X Cbl assay is a microparticle enzyme immunoassay (MEIA) incorporating microparticles coated with porcine intrinsic factor to bind the Cbl.

Preliminary studies were carried out to determine the extent of removal of holo-TCI and holo-TCII from serum by synthetic amor-

phous precipitated silica (Sipernat 283 LS) (PQ Corporation, Valley Forge, Philadelphia, USA). Normal serum was incubated with 2.5 ng ^{57}Co-cyanocobalamin per ml (^{57}Co-CNCbl; specific activity 10 μCi/0.05 μg; Amersham International) at 37°C for one hour and dialysed for 24 hours against phosphate buffered saline (^{57}Co-CNCbl treated serum). Either 30 mg silica powder or 300 μl of an aqueous slurry containing 30 mg silica was added to 1 ml of ^{57}Co-CNCbl treated serum. The mixtures were vortexed, left at room temperature for 10 minutes, centrifuged at 5000 × *g* for 10 minutes and the supernatants removed. Either 800 μl of these supernatants or of silica unadsorbed ^{57}Co-CNCbl treated serum was run on a Sephadex G-200 column as described by Bloomfield and Scott,[9] collecting 4 ml fractions at a flow rate of 12 ml/hour, and the radioactivity in 1 ml of each fraction determined using a Wallac 80000 gamma counter.

To determine holo-TCII concentrations in serum samples, holo-TCII was removed from serum using a slurry containing 6 g silica (Sipernat 283 LS) in 100 ml distilled water; 250 μl of the slurry containing 15 mg silica was mixed with 500 μl serum, vortexed and left at room temperature for 10 minutes. The mixture was then centrifuged at 5000 × *g* for 10 minutes. The supernatant fluid was assayed for Cbl (that is, for holo-TCI and holo-TCIII concentrations) using the IM_X method, allowing for the dilution caused by mixing with the silica slurry (multiplication factor for dilution, ×1.49). Total serum Cbl concentrations were also measured simultaneously using the IM_X method and the holo-TCII concentration calculated by subtracting the holo-TCI plus holo-TCIII concentration from the total serum Cbl concentration.

Results

Figure 1 shows the distribution of radioactivity in the fractions collected by Sephadex G-200 chromatography; the data for the serum treated with silica slurry were corrected for dilution. It is evident that silica did not remove significant amounts of holo-TCI and holo-TCIII (that is, holo-haptocorrin) and removed most of the holo-TCII. In two experiments with silica powder, the percentage removal of holo-TCI was 0.9 and 0.5 and the percentage removal of holo-TCII was 89 and 88, respectively. In one experiment with silica slurry, the removal of holo-TCI and holo-TCII was 0% and 87.5%, respectively.

The precision of total serum Cbl measured by the IM_X method was good, both when assayed in a single run and in different runs, with a coefficient of variation of 3.8% (serum Cbl 355–670 ng/l) and 3.4% (serum Cbl 195–785 ng/l), respectively. The coefficient of variation of Cbl concentrations in aliquots of a single serum sample that were treated separately with silica and assayed in a single run was 4.3%. However, when aliquots of the same serum sample were treated separately with silica and both the untreated and silica treated serum samples were assayed in different runs, the precision for holo-TCII measurements was less satisfactory, with a coefficient of variation of 17.7%; the total Cbl concentration in the serum sample studied in this way was 289 ng/l and its holo-TCII concentration was 43.9 ng/l.

The serum holo-TCII concentrations in the 50 healthy adults were log normally distributed, with a median value of 87 ng/l and 95% reference limits of 12.9–544.7 ng/l. The serum holohaptocorrin concentrations were also log normally distributed, with a median value of 381 ng/l and 95% reference limits of 188–771 ng/l.

The eventual diagnoses in the 30 patients studied were: pernicious anaemia (nine patients); post-gastrectomy Cbl deficiency (one patient); dietary deficiency of folate or folate and Cbl (four patients); chronic alcoholism (four patients); myelodysplastic syndrome (MDS) (five patients); macrocytosis secondary to treatment with methotrexate for rheumatoid arthritis (two patients) or to treatment with azathioprine (one patient); and congenital dyserythropoietic anaemia (CDA) (four patients). In three of the patients with CDA, Cbl and folate independent megaloblastic erythropoiesis was the major abnormality present; the fourth patient had CDA type I.

Fifteen patients had low total serum Cbl concentrations (that is, < 211 ng/l by the IM_X method) and a combination of a low total serum Cbl and a low holo-TCII concentration was found in all patients with pernicious anaemia, all but one of the patients with nutritional deficiency, one patient each with chronic alcoholism or MDS, and one patient with CDA. The combination of a normal total serum Cbl and a low holo TCII concentration was found in three patients with CDA, two patients with chronic alcoholism, one patient each with dietary folate deficiency or MDS, and one of the patients on methotrexate. The combination of a normal total serum Cbl and a normal holo

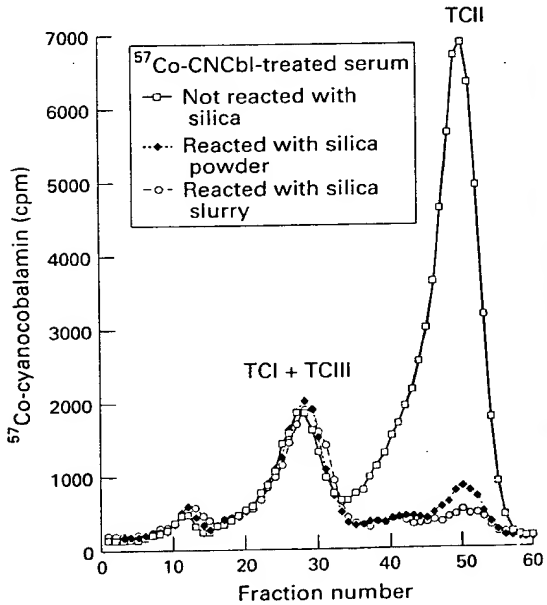

Figure 1 Radioactivity per ml of fractions obtained by Sephadex G-200 chromatography of ^{57}Co-CNCbl treated serum before and after reaction with silica powder or silica slurry.

Table 1 Distribution of normal and high deoxyuridine suppressed values (dU-sv) and normal and low holo-TCII concentrations in the various categories of patients studied

Cause of macrocytosis	Number of cases			
	dU-sv		Holo-TCII	
	Normal	High	Normal	Low
Pernicious anaemia	–	9	–	9
Post-gastrectomy Cbl deficiency	–	1	–	1
Dietary deficiency of folate ± Cbl	–	4	–	4
Chronic alcoholism	4	–	1	3
Myelodysplastic syndromes	5	–	3	2
Methotrexate treatment	1	1	1	1
Azathioprine treatment	1	–	1	–
CDA	4	–	–	4

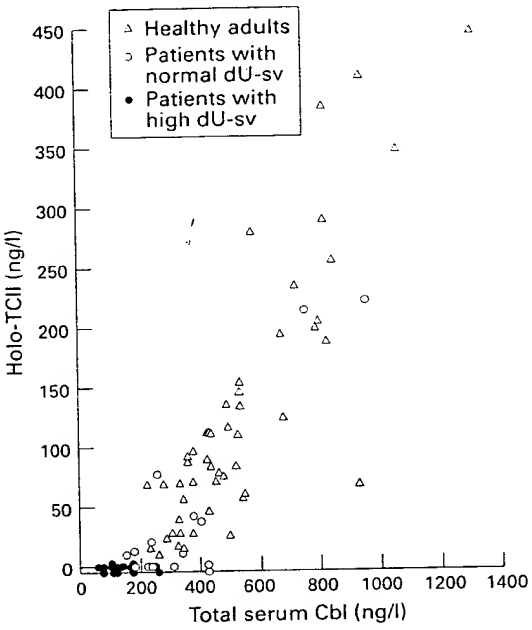

Figure 2 Relation between total serum Cbl and holo-TCII concentrations in 50 healthy volunteers, in 15 patients with normal deoxyuridine suppressed values (dU-sv) and in 15 patients with high deoxyuridine suppressed values.

TCII concentration was found in three patients with MDS, one chronic alcoholic and one patient each on azathioprine or methotrexate.

All of the patients with pernicious anaemia or dietary folate deficiency, the patient with post-gastrectomy Cbl deficiency and one of the two patients on methotrexate had high deoxyuridine suppressed values—that is, they had impaired methylation of deoxyuridylate to thymidylate in their bone marrow cells. The remainder had normal values—that is, had no evidence of impairment of this Cbl and folate dependent reaction. The relation between the deoxyuridine suppressed values and serum holo-TCII concentrations is presented in table 1. It is evident that all 15 patients with high deoxyuridine suppressed values and nine of 15 with normal deoxyuridine suppressed values, including the four patients with CDA, had low holo-TCII concentrations. All but four of the patients with normal deoxyuridine suppressed values and low holo-TCII concentrations had normocellular marrow fragments; the four exceptions were the patients with CDA who had very hypercellular marrow fragments as a result of erythroid hyperplasia.

Figure 2 illustrates the relation between total serum Cbl and holo-TCII concentrations in patients and controls. It is evident that there is a considerable overlap between the results in healthy subjects and patients and between the results in patients with high and normal deoxyuridine suppressed values.

Discussion

Three methodological difficulties were identified in the measurement of serum holo-TCII concentrations by the silica adsorption method. Firstly, the lower limit for the sensitivity of most currently used methods for the measurement of Cbl, including the IM_X Cbl assay, is around 60 ng/l. Holo-TCII assays on serum samples giving a Cbl value for silica adsorbed serum below 60 ng/l will be unreliable. Secondly, treatment with silica does not result in the complete removal of holo-TCII; however, the extent of removal is high, being about 88%. Thirdly, the reproducibility of holo-TCII measurements was relatively poor, partly because holo-TCII measurements are calculated as a difference between two assay results, each with its own coefficient of variation. More accurate holo-TCII measurements on serum samples with a low total Cbl concentration would require the use of the *Euglena gracilis* microbiological assay, which can measure Cbl concentrations accurately down to 20 ng/l.[10]

In the present study, all 10 patients with Cbl deficiency had undetectable concentrations of holo-TCII. However, undetectable or low concentrations were also found in patients with dietary folate deficiency, in one of the two patients on methotrexate and in a mixed group of nine patients with normal deoxyuridine suppressed values in which the diagnoses were chronic alcoholism, MDS or CDA. Thus, whereas a low holo-TCII concentration seems to be a common feature of a degree of Cbl deficiency which gives rise to macrocytosis and a high deoxyuridine suppressed value, it does not help in distinguishing between Cbl deficiency and other causes of macrocytosis.

TCII is synthesised by several cell types including hepatocytes, endothelial cells and ileal enterocytes.[3] As the initial plasma half-life of human holo-TCII injected into humans and rabbits is short (about 60 minutes[11 12]), holo-TCII must be in a very dynamic state. The concentration of holo-TCII in plasma would depend on a complex balance between the rate of removal of Cbl from plasma holo-TCII by various cell types and the rates of entry into the plasma of new pre-formed holo-TCII molecules or generation in the plasma of holo-TCII by the reaction of newly released Cbl with plasma apo-TCII. Newly absorbed Cbl seems to combine with apo-TCII within the enterocyte and enter the circulation as holo-TCII.[3] A low holo-TCII concentration may therefore result from decreased absorption of Cbl in the terminal ileum, a reduced rate of release of Cbl from the liver and other tissues for binding to apo-TCII or an increased rate of clearance of holo-TCII by binding to specific receptors on haemopoietic and other cells, or

by some other mechanism. Previous investigators have only considered the first of these possibilities. Consequently, they have interpreted low holo-TCII concentrations in patients without haematological or biochemical changes attributable to Cbl deficiency as evidence of negative Cbl balance of recent onset due to reduce intake or absorption of this vitamin.[4 5] Our finding of low holo-TCII concentrations in as many as 60% of patients with normal deoxyuridine suppressed values suggests that one or more of the other mechanisms mentioned above may also be important. In particular, the finding of low holo-TCII concentrations in all four patients with erythroid hyperplasia due to CDA, indicates that noticeably increased erythropoietic activity may be an important cause of low holo-TCII concentrations. The poor precision of serum holo-TCII concentrations when measured in different assay runs by current methods must also contribute to the high prevalence of apparently low holo-TCII concentrations.

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